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Anaerobic Studies of Ethylic and Methylic Biodiesel and Impact on Benzene Biodegradation

FRANCISCO SOARES PORTUGAL

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Presidente do Júri: Cidália Maria de Sousa Botelho
(Professora Auxiliar do Departamento de Engenharia Química da Faculdade de Engenharia da Universidade do Porto - FEUP)

Orientador académico: Anthony Steven Danko
(Investigador Auxiliar do Departamento de Engenharia de Minas da Faculdade de Engenharia da Universidade do Porto - FEUP)

Co-orientador académico: Joana Maia Moreira Dias
(Professora Auxiliar Convidada do Departamento de Engenharia Metalúrgica e de Materiais da Faculdade de Engenharia da Universidade do Porto - FEUP)

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Editado por:

FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO

Rua Dr. Roberto Frias

4200-465 PORTO

Portugal

Tel. +351-22-508 1400

Fax +351-22-508 1440

Correio eletrónico: feup@fe.up.pt

Endereço eletrónico: http://www.fe.up.pt

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Resumo

O consumo excessivo de fontes de energia derivadas do petróleo, em particular no sector dos transportes, está na origem de diversos problemas relacionados com a poluição ambiental, tendo também como consequência o preço elevado dos combustíveis. De modo a enfrentar esses desafios, a comunidade internacional tem vindo a promover o desenvolvimento e utilização de fontes de energia renováveis e limpas, como é o caso dos biocombustíveis. De entre os biocombustíveis produzidos atualmente, o bioetanol e o biodiesel apresentam o maior destaque.

O biodiesel é um tipo de biocombustível obtido a partir de fontes de triglicerídeos, como o óleo vegetal, o óleo alimentar residual e a gordura animal. O biodiesel é geralmente utilizado em mistura com o petrodiesel, numa proporção até 30%. O benzeno é um hidrocarboneto monoaromático tóxico, cancerígeno, desloca-se com relativa facilidade e é persistente sob condições anaeróbias. O presente trabalho teve como objetivo estudar a biodegradação de benzeno na presença de biodiesel etílico e metílico, simulando uma situação real de um derrame de combustível contendo simultaneamente petrodiesel e biodiesel. O biodiesel foi produzido por metanólise e etanólise de óleo de girassol virgem e etanólise de óleo alimentar usado, sendo este caracterizado de acordo com a Norma Portuguesa NP EN 14214. Os principais parâmetros de qualidade selecionados foram o teor de água, teor de ésteres de ácidos gordos, índice de acidez, viscosidade cinemática a 40 º C e estabilidade à oxidação a 110 º C.

A biodegradação do biodiesel, do benzeno, e do biodiesel misturado com o benzeno foi determinada por quantificação do metano produzido e da concentração de benzeno nas amostras, por cromatografia em fase gasosa, durante um período de incubação de aproximadamente 60 dias. Os resultados finais mostraram que o biodiesel metílico de óleo virgem de girassol foi facilmente biodegradado por si só e quando misturado com benzeno. Era esperado um impacto negativo do biodiesel relativamente à biodegradação do benzeno de acordo com estudos anteriores. No entanto, apenas se constatou um ligeiro efeito do biodiesel etílico de óleo alimentar usado. Uma vez que este trabalho faz parte de um processo contínuo, conclusões finais só poderão ser tomadas quando a produção de metano cessar.

Palavras-chave: Biodiesel, transesterificação, benzeno, biodegradação.

Abstract

The excessive consumption of petroleum-derived energy sources, especially in the transport sector, has led to issues related to environmental pollution and high fuel prices. In order to address these challenges, the international community has been actively pursuing the development and use of renewable and clean forms of energy like biofuels. An overwhelming majority of biofuels that are currently produced are either bioethanol or biodiesel.

Biodiesel is a type of biofuel obtained from triglyceride sources, such as vegetable oils, animal fats and waste oils or fats. Biodiesel is typically used blended with petrodiesel, at up to 30 %. Benzene is a toxic, carcinogenic, relatively mobile monoaromatic hydrocarbon, and persistent under methanogenic conditions. The present work aims to study the biodegradation of benzene in the presence of ethylic and methylic biodiesel by simulating a real situation of a fuel spill containing both petrodiesel and biodiesel. Biodiesel was produced by methanolysis and ethanolysis of virgin sunflower oil and ethanolysis of waste frying oil, and further characterized according to the Portuguese Biodiesel Standard NP EN 14214. The key quality parameters selected were water content, fatty acid esters content, acid value, kinematic viscosity at 40 ºC and oxidation stability at 110 ºC.

Biodegradation of biodiesel, benzene, and biodiesel blended with benzene were measured by quantifying the amount of methane produced and the concentration of benzene in the samples, by gas chromatography, during an incubation period of approximately 60 days. The final results showed that the methylic biodiesel obtained using sunflower virgin oil was easily biodegraded by itself and when blended with benzene. A negative impact of biodiesel in benzene biodegradation was expected according to previous studies, although only a slight effect with waste frying oil ethylic biodiesel was observed. Since this work is part of an ongoing process, final conclusions can only be taken when methane production is no longer observed.

Keywords: Biodiesel, transesterification, benzene, biodegradation.

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Notation and glossary

B 20	Fuel containing 20 % of biodiesel and 80 % of petrodiesel
c	Constant of the viscometer
C_L	Concentration of benzene in the liquid phase
H_c	Dimensionless Henry`s constant for benzene
m_{biod}	Mass of biodiesel
m_{oil}	Mass of oil
$\bar{t}$	Mean time
P	Atmospheric pressure
R	Universal constant of the ideal gases
T	Temperature
V_G	Volume of gas in the batch reactor
V_L	Volume of liquid in the batch reactor

List of acronyms

AC	Autoclave controls
AV	Acid value
BTEX	Benzene, toluene, ethylbenzene, and xylene
CMB	Castor oil methylic biodiesel
COD	Chemical oxygen demand
COD_t	Theoretical chemical oxygen demand
EC	Fatty acid esters content
NP EN 14214	Portuguese standard for liquid petroleum products: Fatty acid methyl esters (FAME) for use in diesel engines and heating - Specifications and test methods.
FAE	Fatty acid esters
FAEE	Fatty acid ethylic esters

FAME	Fatty acid methylic esters
FFA	Free fatty acids
FID	Flame ionization detector
GC	Gas chromatograph
GHG	Greenhouse gases
IV	Iodine value
KV	Kinematic viscosity
LCFA	Long chain fatty acids
OS	Oxidation stability
PLMB	Pork lard oil methylic biodiesel
SbMB	Soybean oil methylic biodiesel
SEB	Sunflower oil ethylic biodiesel
SMB	Sunflower oil methylic biodiesel
SO	Sunflower oil
TOC	Total organic carbon
VFA	Volatile fatty acids
WFEB	Waste frying oil ethylic biodiesel
WFMB	Waste frying oil methylic biodiesel
WFO	Waste frying oil
WC	Water content

1. Introduction

1.1 World current energy and environment overview

The rapid development of industrial activity and the radical increase in human population had led to the world`s current energy crisis. The consumption of petroleum-derived energy sources, taking into account that approximately 85 % of all petroleum-derived oil produced is consumed in the transportation sector (Kwon et al. 2013), causes cost escalation, fast natural resources depletion and environmental problems including global warming, emission of particulate matter, acid rain, and extreme climatic phenomena.

Thus, the interest in renewable, sustainable (economically, environmentally and socially) and low-carbon energy technologies has been recently growing. Maintaining the current unsustainable exploitation and consumption of natural resources, energy-related greenhouse gas (GHG) emissions will more than double by 2050 (OECD/IEA 2011). GHG emissions lead to global warming and can be reduced by improving energy efficiency and investing in renewable forms of energy, carbon capture and storage, nuclear power, and new transport technologies (Utlu 2007, OECD/IEA 2011, Ang et al. 2013). Focus on renewable energy sources with diversification of sources along with more efficient fuel utilization systems is essential to respond to the limited reserves of fossil fuels. The alternative must be technically practicable, economically competitive, readily available and environmentally acceptable (Hsiao et al. 2012).

1.2 Biofuels

1.2.1 Definition

Biofuels might be defined as liquid fuels derived from biomass including plants, vegetable oils, forest products and waste materials. The raw materials are grown specifically for fuel purposes or can be residues produced from agricultural or urban activities (OECD/IEA 2011).

Bioethanol, biobutanol, biodiesel, biogas and biomethanol are some examples of types of biofuels. An overwhelming majority of biofuels that are produced are either bioethanol or biodiesel.

1.2.2 Environment and social/economic framework

Biofuels currently provide only approximately 2 % of total transport fuel (OECD/IEA 2011). However, new technologies offer considerable potential for growth over the coming decades and it is foreseen that by 2050 biofuels will be used globally providing 27 % of world transport fuel (OECD/IEA 2011).

In order to address this challenge, the European Union (EU) has adopted policies promoting the use and development of biofuels. Member states were required to have at least 10 % renewable fuels (including biofuels) by 2020 (Directive 2009/28/EC) and this value will increase in the following years.

Public and political opinion is divided when it comes to biofuels. For some, biofuels are the solution and a central technology in the fight against climate change. Opponents are against the use of first generation biofuels (biofuels made from edible matter) due to its competition with the food sector. Therefore, a considerable amount of studies have been done to investigate the potential of second generation biofuels (non-food biomass) like the ones obtained using microalgae, jatropha and industrial waste such as woodchips (Bringezu et al. 2009).

This new feedstock is environmental friendly, renewable and might be totally independent from petroleum. However, such a technology can also induce negative environmental impacts, caused for instance by the use of pesticides and fertilizers, and can also create a competition for land use for food crops. In fact, clearing tropical forests for biodiesel production leads to far greater carbon emissions than those saved by substituting biofuel for fossil fuel in vehicles (Lardon et al. 2009, Bringezu et al. 2009, Vonortas and Papayannakos 2014).

Forethought and careful planning can lead to a socially and economically balanced society where human needs, natural biodiversity protection and ecological systems equilibrium are mutually satisfied (Ang et al. 2013). This vision can be achieved by adopting strong and balanced policies including commercialization of advanced biofuel technologies, efficiency improvements and further cost reductions along with the production chain of different biofuels (Dale et al. 2010, OECD/IEA 2011).

1.2.3 Bioethanol and biodiesel

Biofuels (e.g., bioethanol or biodiesel) are now regarded as feasible alternative options for transportation fuels due to their compatibilities with current internal combustion engine technology and distribution networks since any fossil fuel alternative must have similar physical and chemical properties as regular diesel/gasoline to compete against it (Utlu 2007). Biodiesel production cost is lower than the production cost of bioethanol which makes biodiesel a more attractive alternative (Hamelinck et al. 2007). Biodiesel is therefore the current most used fossil diesel alternative being typically used alone or blended with diesel at percentages up to 30 %.

1.3 Biodiesel

1.3.1 Definition and characteristics

Biodiesel is a type of biofuel obtained from triglyceride sources, such as vegetable oils, animal fats and waste oils or fats. The properties of biodiesel depend on the physicochemical properties of feedstock and the production method. Biodiesel has a comparable calorific value to petro-diesel, has shown higher combustion efficiency (therefore less particulate matter, unburnt hydrocarbons and carbon monoxide emitted), excellent lubricity and low pollutant emission. Furthermore, it is biodegradable, presents low toxicity, contributes to the development of rural economies, and the higher flash point makes it safer to handle, transport and store (Maddikeri et al. 2012, Calero et al. 2014).

The current legislation in Portugal (DL nº 6/2012) requires fuel distribution companies to meet a target of 5.5 % of biofuels in fuels. This value will gradually increase and reach 10 % in 2020. In order to meet these demands, biodiesel production in Portugal has significantly increased over the past several years.

Most researchers agree that the use of raw vegetable oils in a diesel engine fuel without modification, even as low as 10 % to 20 %, results in engine power decreases and specific fuel consumption increase, along with maintenance problems responsible for reducing engine lifetime (NREL 2009).

Vegetable oils can be used as fuels after their viscosity, around 40 $mm^2\ s^{-1}$ at 40 ºC (NREL 2009), is reduced to close to 4 to 5 $mm^2\ s^{-1}$ (NREL 2009) by transesterification, which is the most common chemical process to obtain an appropriate substitute of diesel fuel known as fatty acid alkyl esters (biodiesel). While cooking, the viscosity and acidity of the oil generally increases (Utlu 2007) due to degradation reactions caused by high temperatures and the presence of air and water. Under these conditions, the following reactions

occur: hydrolysis due to presence of water which produces Free Fatty Acids (FFA) and mono- and diglycerides; oxidation by contact with oxygen, forming volatile compounds like aldehydes and ketones; and polymerization caused by the combined effect of hydrolysis, oxidation and high temperatures, which reaction products are dimeric and polymeric triglycerides with ring structure (Gertz 2000, Choe et al. 2007, Sanli et al. 2011). Producing biodiesel from waste frying oil might be a challenge when it has a high FFA percentage, requiring a pre-treatment to allow conventional processes to be applied.

1.3.2 Environment and social/economic framework

The higher costs of the feedstock and associated technical challenges related to biodiesel production still lead to a high price of biodiesel, which makes it difficult with respect to economic sustainability without incentives. The continuous and stable supply of energy resources is essential to achieve a sustainable economy development. In order to address this challenge, a circular economy is necessary, leading to a "resources–to–product–to–regenerated-resources" closed loop to minimize resource loss and environmental cost (Wen-bo et al. 2011). Producing biodiesel from waste edible oils and fats is a productive activity following the 3R principle. Waste cooking oil is much less expensive than pure vegetable oil, and, therefore, a promising alternative feedstock (Calero et al. 2014, Dias et al. 2014), especially if stimulated with economic benefits (Zhang et al. 2003).

Fatty Acid Ethylic Esters (FAEE) production is technically and chemically similar to Fatty Acid Methylic Esters (FAME) production. This fact allows the existing plants for the production of FAME to be adapted for the production of FAEE. Although limited information available about the behavior of FAEE in the motors, a similar behavior compared to FAME is expected. FAEE use allows greater energy efficiency. Currently, FAEE cost production is above FAME cost production. However, this scenario can be reversed in the coming years if the methanol costs increase at a higher rate than ethanol (Hamelinck et al. 2007, Dias et al. 2014).

1.3.3 Transesterification reaction

Transesterification is a chemical reaction between a triglyceride (vegetable oil/animal fat) and an alcohol, most commonly methanol or ethanol, in the presence of a catalyst, to form a methylic or ethylic ester and the byproduct glycerol (Rashid et al. 2009, Nayak et al. 2013).

The stoichiometric amount for the transesterification (Figure 1) reaction implies the use of 3 mol of alcohol to 1 mol of triglyceride, resulting in 3 mol of ester and 1 mol of glycerol.

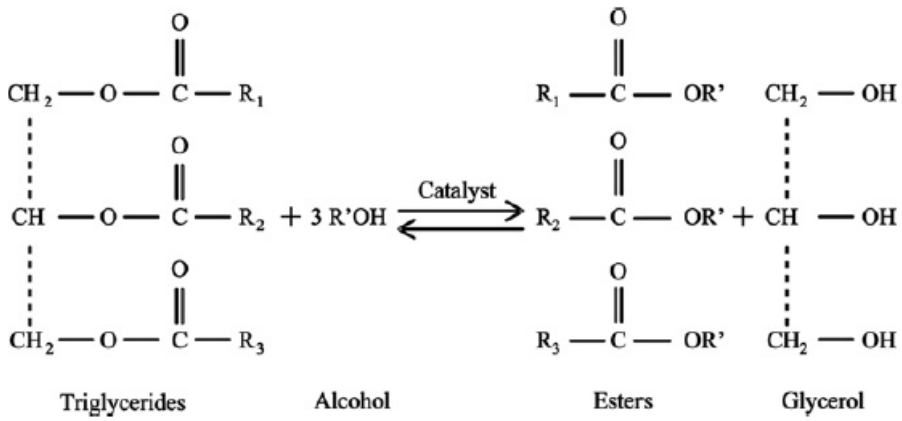

Figure 1. Transesterification reaction of triglycerides. Source: Dias et al. 2008.

Although the transesterification reaction is theoretically reversible, the fact that glycerol is immiscible in biodiesel makes the reverse reaction inexistent or negligible (Knothe et al. 2005).

1.3.4 Biodiesel quality

The quality of the ester produced depends on the effectiveness of the reaction that might be optimized in terms of the molar ratio of alcohol: vegetable oil, the type and amount of alcohol and catalyst, reaction time and temperature, the product purification process, and mostly the quality of the oil, namely its FFA content and water content (Utlu 2007, Rashid et al. 2009). Storage conditions are another factor that affects biodiesel quality.

Excess of alcohol is usually used in order to shift the equilibrium to the products side and, therefore, maximize the esters production. Methanol is the most widely used alcohol due to its low cost and the ability to dissolve alkalis (Nayak et al. 2013). However, methanol is mostly obtained from fossil resources, presents high toxicity, and it may cause blindness and cancer. On the other hand, ethanol is non-toxic, biodegradable and obtained from renewable sources (Knothe et al. 2006), and therefore is considered a “green” and promising alternative to methanol. The catalyst may be an alkyl, acid or enzyme; the alkali catalyzed transesterification is the easiest and the fastest proven route for the reaction. The reaction time must be optimized to lead to the maximum amount of esters produced. Several authors showed that the optimal reaction time is 60 minutes, which leads to a conversion rate of over 95 wt. % according to Dias et al. (2014) study. Optimal temperature is generally close to the one which corresponds to the boiling point of the alcohol used, although when ethanol is used, lower temperatures have found to lead to higher conversions (Dias et al. 2014). In addition, the methanolic route was shown to achieve high product purities at lower temperatures (Moreira et al. 2010).

The saponification reaction is aggravated when using WFO as a raw material due to its high FFA level, leading to operational problems regarding emulsion production through water washing, as well as inhibition or reduction of catalyst performance (due to its consumption) during the base transesterification, causing lower ester yields and purity (Kusdiana and Saka 2004, Gui et al. 2008, Palanisamy et al. 2013). Water washing produces a large amount of waste water contributing to a lower economical and environmental performance. Therefore, studies regarding alternative water-free purification processes have been developed (Güllü and Demirbaş 2001, Dias et al. 2014).

Table 1 shows the limits of the biodiesel quality parameters, according to the Portuguese Standard NP EN 14214, 2014.

Table 1 - Generally applicable requirements and test methods. Source: PORTUGUESE STANDARD NP EN 14214, 2014.

Property	Unit	Limits		Test Method
		Minimum	Maximum	
FAME content	% (w/w)	96.5	-	EN 14103
Density at 15 ºC	kg/m^3	860	900	EN ISO 3675 EN ISO 12185
Kinematic viscosity at 40 °C	mm^2/s	3.50	5.00	EN ISO 3104
Flash Point	ºC	101	-	EN ISO 2719 EN ISO 3679
Sulfur content	mg/kg	-	10.0	EN ISO 20846 EN ISO 20884 EN ISO 13032
Cetane number	-	51.0	-	EN ISO 5165
Sulfated ash content	% (w/w)	-	0.02	ISO 3987
Water content	mg/kg	-	500	EN ISO 12937
Total contamination	mg/kg	-	24	EN 12662
Copper strip corrosion (3 h at 50 ºC)	Rating	Class 1		EN ISO 2160
Oxidation stability at 110 °C	h	8.0	-	EN 14112 EN 15751
Acid value	mg KOH/g	-	0.50	EN 14104
Iodine value	g iodine/100 g	-	120	EN 14111 EN 16300
Linolenic acid methylic esters	% (w/w)	-	12.0	EN 14103
Polyunsaturated (≥ 4 double bonds) methylic esters	% (w/w)	-	1	EN 15779
Methanol content	% (w/w)	-	0.20	EN 14110
Monoglyceride content	% (w/w)	-	0.70	EN 14105
Diglyceride content	% (w/w)	-	0.20	EN 14105
Triglyceride content	% (w/w)	-	0.20	EN 14105
Free glycerol	% (w/w)	-	0.02	EN 14105 EN 14106
Total glycerol	% (w/w)	-	0.25	EN 14105
Group I metals (Na + K)	mg/kg	-	5.0	EN 14108 EN 14109 EN 14538
Group II metals	mg/kg	-	5.0	EN 14538
Phosphorous content	mg/kg	-	4.0	EN 14107 FprEN16294

The use of biodiesel with high viscosity can cause problems arising from the loss of combustion efficiency leading to the formation of deposits. However, the viscosity of the biodiesel must be sufficiently high to avoid engine power loss (NREL 2009). High viscosity values are due to the presence of Long Chain Fatty Acids (LCFA) (compounds containing high number of carbon atoms) and is usually aggravated by an increasing degree of saturation. Viscosity of the ethylic esters is slightly higher than the viscosity of the methylic esters (Knothe et al. 2005).

Acid value is a parameter associated with the presence of FFA which results from natural degradation of oils and fats, hydrolysis and oxidation of the biodiesel product. High acid values (greater than 0.50 mg KOH g^{-1}) contribute to the creation of fuel system deposits and lead to the decrease in the lifetime of pumps and filters. Acid value increases when the production process of biodiesel is inappropriate and when transportation/storage conditions lead to its degradation (NREL 2009).

When a transesterification reaction is not complete, tri-, di- , and monoacylglycerols are present in the biodiesel (Knothe et al. 2005), which, combined with glycerol, lead to water emulsification. It is important that, during the transesterification reaction, compounds are free of moisture due to the potential hydrolysis of the esters, producing FFA which causes acid value and viscosity of biodiesel to increase (Knothe et al. 2005).

For the same reason, acid value and viscosity of the oil and biodiesel increase (leading to a change from the original usually yellow color to a darker, brown color) due to the occurrence of hydrolysis processes when water is present during storage (Knothe et al. 2005).

The water present in biodiesel may be dissolved or suspended. It is essential that biodiesel remains dry during storage. However, keeping biodiesel dry is challenging since condensate water is often found at the base of the reservoirs.

The suspended water causes corrosion problems related to the water injection system of the engine. The water issue is also related to the growth of microorganisms which can result in increased acid value of the biodiesel and clogging of filters (Knothe et al. 2005).

Biodiesel can be oxidized during storage and transport forming peroxides, acids, gums and deposits. A minimum value of the oxidation stability is specified so that the biodiesel maintains its physical-chemical properties during storage (NREL 2009). Higher corrosion resistance leads to better quality of biodiesel throughout its lifecycle (Grbic-Galic and Vogel 1987, Domingos et al. 2007, Candeia et al. 2009). The storage stability depends on the storage conditions and time (Joshi et al. 2013).

Glycerol is largely insoluble in the biodiesel facilitating their removal by decantation or centrifugation. The total glycerol is the sum of bound glycerol with free glycerol. Most of glycerol is removed by water washing process, particularly if hot water is used (Knothe et al. 2005).

The bound glycerol includes monoacylglycerols which are formed from glycerol and fatty acids. Free glycerol corresponds to the fraction that is suspended and the residual amount dissolved in biodiesel which may increase when alcohol is present. High concentration of free glycerol in the biodiesel leads to glycerol sedimentation in the tank creating a highly viscous mixture, which can cause clogging of filters and difficulties in engine performance (Knothe et al. 2005).

The properties described are considered critical since they allow assessing the quality of the production process and product.

1.4 Production and release of fuels

Biodiesel is a mixture of monoalkyl esters of saturated and/or unsaturated LCFA (Aktas et al. 2010). The combination and percentage of Fatty Acid Esters (FAE) is a function of the raw material which yields biodiesel (Corseuil et al. 2011).

Diesel is a complex mixture of a variety of hydrocarbons, including monoaromatics. The main monoaromatic hydrocarbons are benzene, toluene, ethylbenzene and xylene isomers (BTEX). Although the percentage of monoaromatic hydrocarbons in fuel represents only 2 to 8 % (Nales et al. 1998), these compounds are of special interest due to their high persistence and toxicity.

The potential risks to the environment and human health arising from the release of biofuels and biofuel/fuel mixtures have received limited attention. These depend on the characteristics of the site and amount and type of biofuel. The study of the physical-chemical properties of the biofuels, in particular the viscosity and/or density, is relevant since they influence its fate and transport and, thus, have an important role in determining its impact on soil and water (EPA 2011, Corseuil et al. 2011).

Biodiesel tends to remain in the area of contamination since it is poorly miscible in water (Corseuil et al. 2011). In addition, dispersion of a contamination plume is usually diminished due to occurrence of aerobic and anaerobic biodegradation. The behavior of biodiesel towards degradation is not well known although the chemical properties of biodiesel are well documented (Aktas et al. 2010). More information concerning the biodegradation of biodiesel and benzene is discussed in the next sections.

1.4.1 Aerobic and anaerobic biodegradation – general considerations

Microbial degradation allows for the complete mineralization of hazardous substances and therefore is considered the most attractive removal process. Studies conclude that the microbial density is higher in contaminated soils with hydrocarbons, compared to unpolluted soil (Rakoczy et al. 2011).

The energy required for the maintenance and growth of microbial population is obtained from energy released from redox processes as a result of transfer of electrons from one compound to another (Angelidaki and Sanders 2004). Oxygen levels are replaced in soil, including anaerobic environments, through the oxygen carried by the water entering the system, oxygen released by roots of plants and by air-soil diffusion. Studying the availability of oxygen is important since it is the most efficient electron acceptor of the microbial respiration process (Nales et al. 1998).

Biodegradation of organic contaminants is affected by several factors: bioavailability, contaminants concentration, redox potential, chemical complexity of the compound, oxygen concentration, temperature, pH, moisture, salinity, recalcitrance, presence of micro and macro nutrients that are used by the enzymes, proteins, etc. (Takashima and Speece 1990), and inhibitors (e.g. LCFA) (Prenafeta-Boldú et al. 2002, EPA 2011). Several studies suggest that the addition of nutrients increases microbial activity by 50 % (Kayhanian and Rich 1995, Jansen et al. 2007, Feng et al. 2010, Monanakrishna et al. 2010).

The importance of anaerobic biodegradation lies in the wide variety of microorganisms present in underground environments (soil, aquifers, reservoirs and transport pipelines for petroleum fuels) that are capable of degrading organic compounds (energy sources) under these conditions. The creation of anaerobic conditions is due to low oxygen solubility and the use of readily degradable substrates by aerobic microorganisms leading to faster oxygen consumption than its reset rate. However, the addition of electron acceptors such as Fe (III) oxides, nitrate or sulfate can stimulate anaerobic oxidation of

pollutants. Reductions due to the sulfate and nitrate enable more energy production than methanogenic reductions. Anaerobic biodegradation occurs at a lower rate when compared to aerobic biodegradation due to minor release of energy (Silva and Alvarez 2004).

Optimum pH depends on the type of enzyme and affects solubility/bioavailability of the substrate. Anaerobic digestion occurs at a pH between 6 and 8.3 (Egland et al. 1997, Nales et al. 1998, Angelidaki and Sanders 2004).

1.4.2 Benzene biodegradation

Benzene can be degraded into non-harmful compounds under aerobic or anaerobic conditions. Other natural attenuation mechanisms only move the contaminants, including adsorption, volatilization and dilution (Egland et al. 1997).

Aerobic biodegradation of hydrocarbons is well known (Fisher et al. 1993, Holden and Fierer 2005). The fate of benzene is controlled by the presence of oxygen. Aerobic conditions lead to high rate degradation by microorganisms which are well disseminated in the environment (Carsten et al. 2011). Oxygenase activity (which initiates biodegradation) is detected at oxygen concentrations below 0.1 mg/L (Corseuil et al. 2011).

Oxygen is the electron acceptor, while benzene is the electron donor and is simultaneously a source of carbon and energy, resulting in bacterial growth (Jason et al. 2000). Aerobic biodegradation of benzene, such as any aerobic hydrocarbon degradation, produces carbon dioxide and water as final products.

When oxygen is present, mono- or dioxygenases are produced by benzene degrading organisms which are responsible for activating the aromatic nucleus by molecular oxygen introduction. Thereby, phenol or cis-benzene dihydrodiol are produced and subsequently oxidized to catechol which is broken by dioxygenases in ortho- or meta- position (Carsten et al. 2011).

Less information is available for the anaerobic biodegradation of benzene. Benzene can be degraded anaerobically through different terminal electron accepting processes such as methanogenesis, nitrate-reducing, iron-reducing and sulfate-reducing environments (Silvia et al 2003). Although this is the case, many studies indicate its recalcitrance under methanogenic conditions and that it may require an extended period of adaptation, often years (Grbic-Galic and Vogel 1987, Edwards and Grbic-Galic 1992, Silva and Alvarez 2002).

However, benzene alkylated derivatives, toluene, ethylbenzene and xylene isomers are considered to be less persistent in anoxic environments compared to benzene. The reasons for benzene recalcitrance are not yet known. One possible explanation is that anaerobic organisms which degrade benzene are poorly disseminated in the environment. In addition, a restricted range of microorganisms which require specific and limited environmental conditions for optimum function is needed. This is a possible cause for benzene persistence at some sites or even in laboratory studies using enriched cultures (Vogt et al. 2011). Carsten et al. (2011) studied anaerobic benzene biodegradation and suggested that, in some strictly anaerobic and enriched cultures, benzene was described as being mineralized cooperatively by two or more different organisms while all use nitrate as an electron acceptor.

There are several hypotheses proposed for the first step of the anaerobic degradation of benzene, namely hydroxylation to phenol, carboxylation to benzoate, fumarate addition to the succinyl derivative, reduction, and methylation to toluene. Results of anaerobic biodegradation of benzene tests suggest that carboxylation and hydroxylation of benzene are early steps of the initial reaction mechanisms since benzoate and phenol are constantly detected as intermediates of benzene anaerobic degradation in methanogenic, sulfate-reducing and iron-reducing enrichment cultures (Chakraborty and Coates 2005, Ulrich et al. 2005).

1.4.3 Biodiesel biodegradation

Biodiesel is a chemical compound with a simple structure, which characteristics depend essentially on the type of raw material (sunflower oil, waste frying oil, etc.) and the type of alcohol used (methanol and ethanol), when considering the most common process, transesterification.

In general, biodiesel has been found to be readily biodegradable under aerobic or anaerobic conditions. When released to the environment, biodiesel consumes oxygen and stimulates the proliferation of microorganisms (EPA 2011). Previous studies that compared the aerobic biodegradation of ethylic and methylic esters, which present similar viscosities, suggest that under aerobic conditions, the feedstock of biodiesel had no influence on biodiesel biodegradation (Zhang et al. 1998, Knothe et al. 2005).

Anaerobic microorganisms can readily hydrolyze and convert biodiesel into intermediate fatty acids, regardless of previous exposure to hydrocarbon or biodiesel (Aktas et al, 2010). This may lead to the production of large amounts of methane (potentially an explosive hazard risk), biomass and intermediate Volatile Fatty Acids (VFA).

Anaerobic microorganisms hydrogenate the unsaturated fatty acids (present in biodiesel) to saturated fatty acids before beta oxidation (Sousa et al. 2009). These fatty acids may be quickly biodegraded by indigenous microorganisms although they are sufficiently soluble in water to potentially move away from the contamination source (Corseuil et al. 2011).

Several studies that used soybean, castor and pork lard (via methanol) suggest that unsaturated, less viscous and highly bioavailable biodiesel tend to be more easily degraded (Corseuil et al. 2011, Borges et al. 2014). The viscosity of the biodiesel increases when it is biodegraded due to the formation of oxidized compounds, sediments and gums (Das et al. 2009). It is also affected by the number of double bonds.

Only one study was found regarding methane production – the end product under methanogenic conditions – from biodiesel biodegradation (Borges et al. 2014). The pork lard, castor and soybean biodiesel used in the mentioned tests were produced through methanolic route. No studies were found using ethanol.

1.4.4 Biodegradation of benzene blended with biodiesel

Biodegradation of biodiesel and its impact on the natural attenuation of benzene is complex. The potential impacts of biodiesel-benzene spills on groundwater quality are a function of the composition of biodiesel and spill scenario (Corseuil et al. 2011).

Several studies show that the presence of biodiesel negatively impacted benzene or toluene biodegradation (Corseuil et al. 2011, Borges et al. 2014). The various types of methylic biodiesel were produced from soybean, castor and pork lard oil. Complete toluene removal increased from 25 days to 34 days in the presence of soybean oil biodiesel (Corseuil et al. 2011). Similarly, 45 % of benzene was removed within 34 days with soybean oil biodiesel while 90 % was removed in its absence (Corseuil et al. 2011). Approximately 18 % of benzene was removed after 150 days compared to approximately 15 %, 11 %, and 7 % in the presence of pork lard, soybean and castor oil biodiesel, respectively (Borges et al. 2014). The behavior of other types of biodiesel (including those produced from ethanol) and their effect on anaerobic benzene remediation are not yet known.

One recent study examined the use of ammonium acetate to stimulate biodegradation at a B20 site in the field (Ramos et al. 2013). Ammonium acetate stimulated anaerobic conditions and BTEX removal began after 8 months while removal was not observed in the control plot after two years. The author’s attributed the effect of ammonium acetate on the enhanced growth of BTEX degraders and associated microbial populations that consume acetate and hydrogen and enhanced thermodynamics.

In anaerobic environments, microorganisms often associate themselves together to mutually benefit from biodegradation processes (Sousa et al. 2009, McInerney et al. 2009).

2. Objective

The objectives of this work were to determine the biodegradability of ethylic and methylic biodiesel and compare their impact on benzene biodegradation. In order to address these objectives, the dissertation is divided in three phases: 1) production of three types of biodiesel, two from commercial sunflower oil (SO), through methanolic (SMB) and ethanolic (SEB) routes, and one from a waste frying oil (WFO) through an ethanolic route (WFEB); 2) Characterization of the key quality properties of both types of biodiesel according to NP EN 14214; and 3) biodegradation of biodiesel, benzene, and biodiesel blended with benzene by quantifying the amount of methane produced and the concentration of benzene in the samples by gas chromatography.

3. Materials and methods

3.1 Biodiesel production and characterization

3.1.1 Raw materials

Raw materials inventory

The commercial sunflower oil (SO) used was from the brand "3 ás equilíbrio" and the waste frying oil (WFO) was provided from the FEUP system for collecting waste oils from domestic source.

The biodiesel production process required the use of alcohol, ethanol (P.A., Panreac) and methanol (Fisher Chemical), and a catalyst, NaOH (sodium hydroxide powder 98 %, Sigma-Aldrich, Reagent Grade). A HCl 0.5 % (V/V) solution was used to purify the three types of biodiesel. All the reagents used during synthesis and quality evaluation were of analytical grade.

The determination of the Acid Value (AV) required a KOH solution and a 1:1 (V/V) of diethyl ether (Sigma-Aldrich) and ethanol absolute solution (P.A., Panreac) used as reagent. The CombiCoulomat frit (Merck) reagent was used to determine the Water Content (WC) of the oils and the samples corresponding to the three types of biodiesel. Determining the FAE content required heptane (analytical grade, Merck), and both methyl heptadecanoate (Fluka) and ethyl pentadecanoate (Aldrich) standards, for methylic and ethylic biodiesel, respectively.

Sunflower oil and waste frying oil pre–treatment and characterization

The WFO was pre-treated using vacuum filtration first and then dehydration by evaporation (during 30 min at 90 ºC) in order to remove impurities and potential

residual water which might exist. Figure 2 shows the stages of the pre-treatment process.

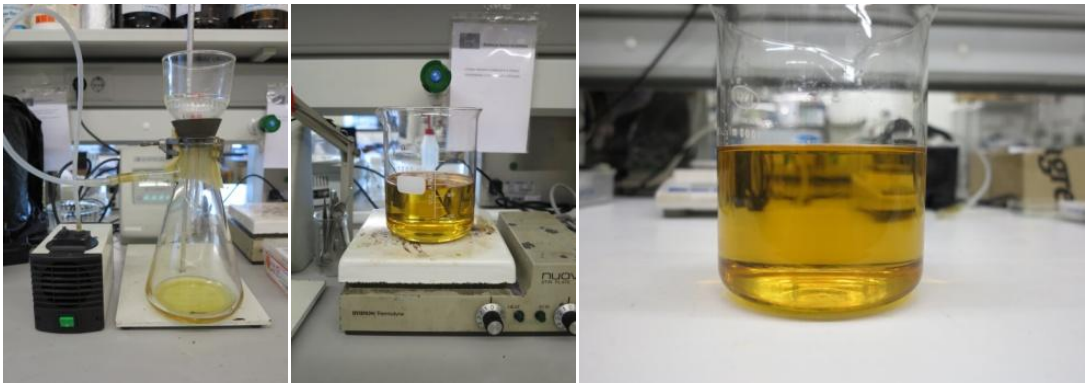

Figure 2. Vaccum filtration system (left), dehydration system (middle), and pre-treated waste frying oil (right).

Both SO and WFO were characterized taking into account their AV and WC. The Iodine Value (IV) could be inferred by analyzing the biodiesel composition in the GC as shown in the section *4.3.2*.

The AV was determined by volumetric titration with KOH using a 1:1 (V/V) of diethyl ether and ethanol solution as reagent (Figure 3) according to NP EN ISO 660. The weight of sample used was 20 g. Equation 1 allows the determination of the AV (mg KOH g^{-1}) based on the volume of the KOH standard solution used for the titration.

$$AV = \frac{M_{KOH} \times V_{KOH} \times c_{KOH}}{m} \quad (1)$$

where M_{KOH} is the molecular weight of KOH solution (g mol^{-1}), V_{KOH} is the volume of the KOH solution (mL), c_{KOH} is the concentration of the KOH solution (mol L^{-1}) (Appendix A1), and m is the sample weight (g).

Figure 3. Volumetric titration system.

WC determination was carried out using a coulometric Karl Fisher titrator (Figure 4) which determines the WC by coulometry according to EN ISO 12937.

Figure 4. Karl Fisher equipment.

The water on biodiesel injected (close to 1 mL) reacts with the CombiCoulomat frit reagent, consuming the iodine present; titration ends when iodine is detected in the vessel (meaning that all the water was consumed). The exact amount of the biodiesel injected was calculated by weighting the syringe before and after the injection. After completing the titration, the values were introduced in the equipment which automatically indicated the WC of the sample.

3.1.2. Biodiesel production

Transesterification reaction

The conditions for the production of sunflower oil ethylic and methylic esters and waste frying oil ethylic esters were selected based on previous studies (Dias et al. 2008, 2014). The fundamental reagent characteristics and reaction conditions used are presented in Table 2. Initially, 200 g of the oil were introduced in a three-necked batch reactor which was immersed in a temperature controlled water bath with magnetic stirring. After stabilizing the temperature, the catalyst dissolved in alcohol was added to the reactor. The transesterification reaction was carried out under atmospheric pressure. In order to avoid loss of alcohol into the gaseous phase, a water-cooled condenser system was used. Once the reaction was completed, the products were transferred to a separatory funnel where the two phases (biodiesel and glycerol) were separated.

Table 2 - Fundamental reagent characteristics and reaction conditions used for biodiesel production (Dias et al. 2008, 2014).

Parameter		**Raw material**		
		SO		**WFO**
Catalyst	Chemical formula	NaOH		
	Concentration (wt. %)	1	0.6	1
	Purity (wt. %)	97		
Alcohol	Chemical formula	Ethanol: CH_3CH_2OH	Methanol: CH_3OH	Ethanol: CH_3CH_2OH
	Purity (wt. %)	Absolute	99.99	Absolute
	Boiling point (ºC)	78	65	78
Raw material	Weigh (g)	200		
Alcohol: raw material molar ratio		6: 1		
Reaction conditions	Reaction temperature (ºC)	45	60	45
	Reaction time (min.)	60	60	60
	Phase separation time (min.)	45		
	Magnetic stirring	4 (in a scale from 0 to 7)		

Figure 5 shows an example of a transesterification reaction system and the separatory funnel containing the ester and the glycerol phases.

Figure 5. Transesterification reaction (left) and biodiesel/glycerol separation by settlement (right).

Biodiesel purification

Biodiesel purification was started by removing the excess alcohol from the biodiesel and glycerol by evaporation using a rotary evaporator, as shown in Figure 6, in agreement with Dias et al. (2008, 2014). Subsequently, biodiesel was washed first with an HCl solution and afterwards with distilled water repeatedly, and then finally dehydrated, also in agreement with the revised studies.

Figure 6. Rotary evaporator.

In agreement with the characteristics of each biodiesel purified, sunflower oil ethylic biodiesel (SEB) was washed sixteen times, sunflower oil methylic biodiesel (SMB) was washed eighteen times, and waste frying oil ethylic biodiesel (WFEB) was washed twenty two times.

3.1.3 Biodiesel characterization

Kinematic viscosity at 40 ºC

The biodiesel kinematic viscosity (KV) at 40 ºC was determined using a capillary viscometer (Cannon-Fenske Routine Viscometer), according to EN ISO 3104. The samples (10 mL) were introduced in the viscometer, immersed in a bath at 40 °C (Figure 7) and temperature-stabilized for around 20 minutes. The time required for the fluid to flow in the tube considering two referenced marks was determined. The viscosity is calculated according to the following expression (Equation 2):

$$KV = c * \bar{t} \quad (2)$$

where KV is the viscosity, in $mm^2\ s^{-1}$, c is the constant at 40 ºC associated to each viscometer, in $mm^2\ s^{-2}$, and $\bar{t}$ is the mean time between three experiments, in seconds.

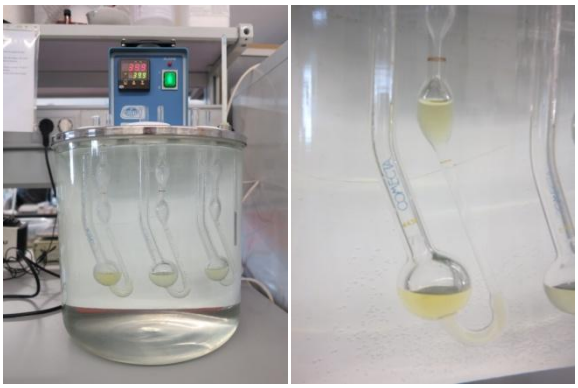

Figure 7. Viscometer bath (left) and Cannon-Fenske Routine Viscometer (right).

Acid value

The AV was determined by volumetric titration according to NP EN ISO 660; the method was identical to the one referred in the section *Sunflower oil and waste frying oil pre-treatment and characterization*.

Water content

The WC was determined according to EN ISO 12937, using the same procedure mentioned in the section *Sunflower oil and waste frying oil pre-treatment and characterization*.

Fatty acid esters content

The Ester Content (EC) (methyl or ethyl) was determined by gas chromatography using a Flame Ionization Detector (FID) according to EN 14103, which relates to methyl EC determination. The Gas Chromatograph (GC) used was a Dani GC 1000 DPC (DANI Instruments S.p.A.) (Figure 8). Methyl heptadecanoate was used as internal standard for methylic esters whereas ethyl pentadecanoate was used as internal standard for ethylic esters. The samples were prepared in duplicate. The volume of sample injected was 1 µL. N_2 was used as carrier and auxiliary gas. The GC consisted on the following: a capillary column injection system with split-flow maintaining the injector at 250 °C; the temperature program initiates the analysis at 120 °C, increasing 4 °C per minute until 220 ºC, and holding at that temperature for 10 min; a AT-WAX (Heliflex capillary, Alltech) column of about 30 m length, 0.32 mm internal diameter and 0.25 µm thick film was used; and a flame ionization detector was maintained at 255 °C. The analysis time was 40 minutes. A data acquisition system and software provided each chromatogram and respective analysis.

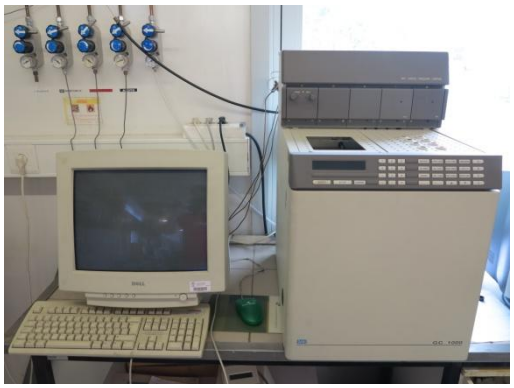

Figure 8. Gas chromatograph, Dani GC 1000 DPC.

Oxidation stability at 110 ºC

The Oxidation Stability (OS) of the FAE was determined using a Rancimat equipment (Figure 9) based on the standard EN 14112: 2003.

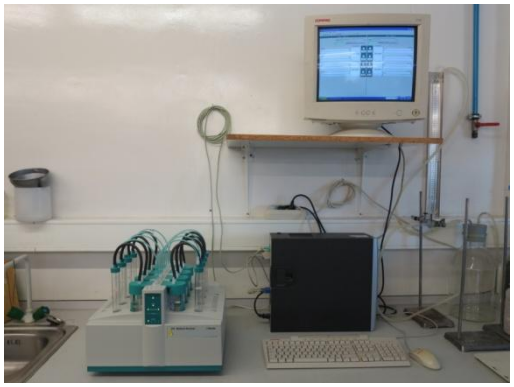

Figure 9. Rancimat equipment.

The data acquisition system records the conductivity of an aqueous solution containing the degradation products, which result from continuously passing air through the biodiesel sample. The analysis is performed at 110 °C. Figure 10 shows an example of the graphical representation of the OS determination.

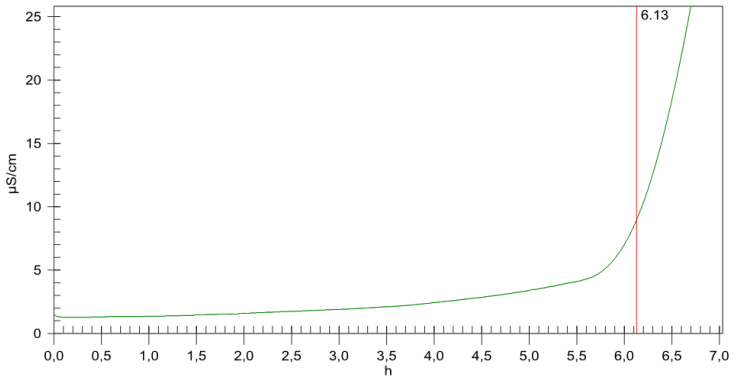

Figure 10. Illustrative example: graph resulting from the determination of the oxidation stability of a sunflower oil sample.

3.2 Biodegradation tests - experimental setup

Anaerobic batch biodegradation tests were setup in 160 mL serum bottles using approximately 17 g of sediment, collected from Estarreja, Portugal (Figure 10), along with 100 mL of basal minimal media (Appendix B5), which contains bicarbonate, micro and macronutirents, salts and resazurin (a redox indicator). The total volume of liquid and sediment was approximately 123 mL.

Figure 11. Site for sediment sampling (Estarreja, Portugal).

Before starting the tests, the batch reactors were purged with nitrogen (approximately 450 mL/min) for 30 minutes and then sealed with Teflon©-lined septa and aluminum crimp caps. Afterwards, approximately 7.4 mL of CO_2 was injected in each bottle, in order to bring the pH down to approximately 7.2-7.3. The Total Organic Carbon (TOC) content of the sediment was 4.3 wt. %. Inorganic carbon was not detected which means that the organic carbon was equal to the total carbon. The experimental design included the following treatments: biodiesel alone and biodiesel + benzene for the three types of biodiesel produced. Autoclave Controls (AC), which were autoclaved 3 times at 121 ºC for 1 hour (3 hours total), were also setup to assess abiotic losses. A total of 36 anaerobic batch reactors were prepared: 20 for the live samples and the remaining 16 for the AC. The microcosms were incubated quiescently and upside down (ensuring that liquids and solids were in contact with the septum). Since light and high temperature accelerates FAE oxidation, the batch reactors were maintained at dark and room temperature (20-25°C).

The bottles were setup (day 0) based on the following composition:

1. Control test
2. 60 mg/L of SEB
3. 60 mg/L of SMB
4. 60 mg/L of WFEB
5. 0.9 mg/L of Benzene
6. 60 mg/L of SEB and 1.7 mg/L of Benzene
7. 60 mg/L of SMB and 1.7 mg/L of Benzene
8. 60 mg/L of WFEB and 1.7 mg/L of Benzene
9. Control test (AC)
10. 60 mg/L of SEB (AC)
11. 60 mg/L of SMB (AC)
12. 60 mg/L of WFEB (AC)
13. 1.6 mg/L of Benzene (AC)
14. 60 mg/L of SEB and 2.3 mg/L of Benzene (AC)
15. 60 mg/L of SMB and 2.1 mg/L of Benzene (AC)
16. 60 mg/L of WFEB and 2.5 mg/L of Benzene (AC)

Tests 1-4 and 9-16 were setup in duplicate, while tests 5-8 were setup in triplicate. A control test (without adding benzene or biodiesel) was necessary to assess the amount of methane produced due to the biodegradation of the organic matter present in the sediment. The amount of Chemical Oxygen Demand (COD) added from biodiesel corresponds to approximately 150 mg/L and can theoretically produce approximately 6.1 mL of methane. This amount of methane production is less than the total headspace volume (37 mL) in the microcosms. Therefore, it was not necessary to depressurize the serum bottles because of gas production. The calculation method of the theoretical methane production due to biodiesel biodegradation is explained in Appendices B1 and B2.

A GC (Shimadzu GC-2014) (Figure 12) equipped with a Flame Ionization Detector (FID) was used for the analysis of methane and benzene. The temperatures used were as follows: injector (200°C), detector (200°C), and column (isothermal at 175 ºC). The amount of sample injected was 0.1 mL of headspace. Tests were performed to study the evolution of the production of methane and the concentration of benzene. The volume of methane produced was calculated based on the ideal gas equation, assuming a pressure of 1 atm and a temperature of 25ºC (Appendix B3), and the concentrations of benzene in the aqueous solution were determined using Henry's constant (Appendix B4). The output of the GC showed two major peaks which corresponded to methane (visible after 0.5 min) and benzene (visible after 3-4 min). The first analysis regarding methane production and benzene concentration took place 6 days after the setup of the live batch reactors, and 4 days after for the AC. Samples were monitored for methane production and benzene losses for approximately 60 days.

Figure 12. Gas chromatograph, Shimadzu GC-2014.

Although beyond the scope of this work, samples were also periodically removed from both live and autoclaved bottles for future microbial community and biodiesel biodegradation intermediates analyses. The volume extracted for each sample at each sampling point was 5 mL (1 mL for microbial analysis and 4 mLs for LCFA). Two calibration curves for methane and benzene were needed in order to normalize the changes in the liquid to headspace ratio due to the removal of liquid samples.

4. Results and discussion

4.1 Raw materials characterization

Figure 13 shows the visual appearance of SO and WFO before the transesterification reaction.

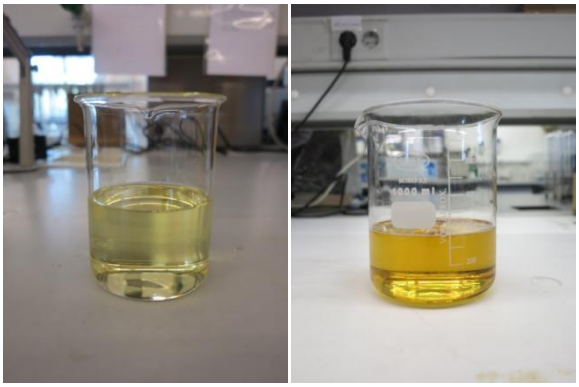

Figure 13. Sunflower oil (left) and pre-treated waste frying oil (right).

Table 3 shows the main properties of the raw materials.

Table 3 - Acid value, water content and oxidation stability at 110 ºC of sunflower oil and waste frying oil.

Parameter	Oil	
	SO	WFO
AV (mg KOH g^{-1})	0.0722	0.407 ± 0.002
WC (mg kg^{-1})	427 ± 13	487 ± 11
OS (h)	6.125 ± 0.006	1.56 ± 0.02

The low AV of SO is typical of refined oils (Tawde et al. 2013). The AV is higher for the WFO as expected due to chemical reactions that occur during the frying

process; although a much higher AV has been reported for WFO. The WC value is similar for both oils due to the pre-treatment performed. The OS value is significantly higher for SO, being a virgin oil. The darker color of the WFO is a reflection of the degradation/alteration of the oil during the frying/storage process, which leads to its lower OS (Knothe et al. 2005).

4.2 Product yield

The Product Yield (PY) (wt. %) is one of the main parameters which represent the efficiency of the biodiesel producing process, being obtained from the following expression (Equation 3):

$$Product\ yield = \frac{m_{biod}}{m_{oil}} \times 100 \quad (3)$$

where m_{biod} is the weight of the purified biodiesel and m_{oil} is the mass of pre-treated oil.

Table 4 shows the PY obtained.

Table 4 - Product yield of sunflower oil ethylic biodiesel, sunflower oil methylic biodiesel and waste frying oil ethylic biodiesel.

Biodiesel	SEB	SMB	WFEB
PY (wt. %)	73.0	95.3	69.6

The PY is known to be higher when virgin oils are used as raw materials rather than waste oils (Moreira et al. 2010). A previous study by Dias et al. (2014) showed that while using conventional washing, PY of SEB and WFEB were

90.5 wt. % and 85.6 wt. %, respectively. In another study by Velez et al. (2012), the PY obtained for SEB value was 90.0 wt. %. Dias et al. (2008) results showed that the PY of SMB was higher (reaching 97.0 wt. %) than the value corresponding to WFMB (reaching 92.0 wt. %) (Dias et al. 2008). The study by Rashid et al. (2009) regarding the production of SMB, showed that, using the optimal transesterification conditions studied, a PY of 97.8 wt. % was achieved.

As expected, the PY was higher for SMB due to the ability of methanol to dissolve alkalis (Nayak et al. 2013). The literature confirms the relation between the three types of biodiesel: PY of SMB is higher than PY of SEB (Georgogianni et al. 2007), and the PY of the sunflower oil biodiesel is higher than the one of waste frying oil biodiesel when both are produced through the same route (methanol or ethanol) (Dias et al. 2008, 2014). The lowest purity values of SEB and WFEB are related to the substantial amount of emulsion produced during the water-washing phase (Figure 14). Emulsification is known to cause problems regarding separation of waste water and biodiesel, which may lead to greater biodiesel losses compared to SMB (Kusdiana and Saka 2004, Gui et al. 2008, Palanisamy et al. 2013). In addition, WFO contains impurities which might lead to lower PY values (Maddikeri et al. 2012).

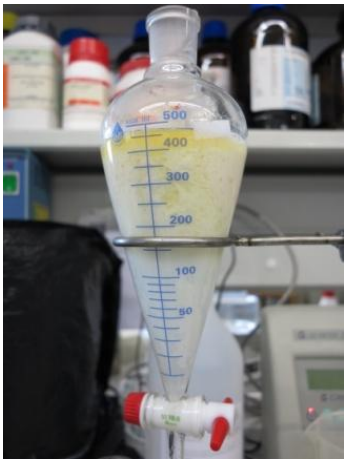

Figure 14. Water-washing process of waste frying oil ethylic biodiesel.

Figure 15 shows the final look of SEB, SMB and WFEB after purification.

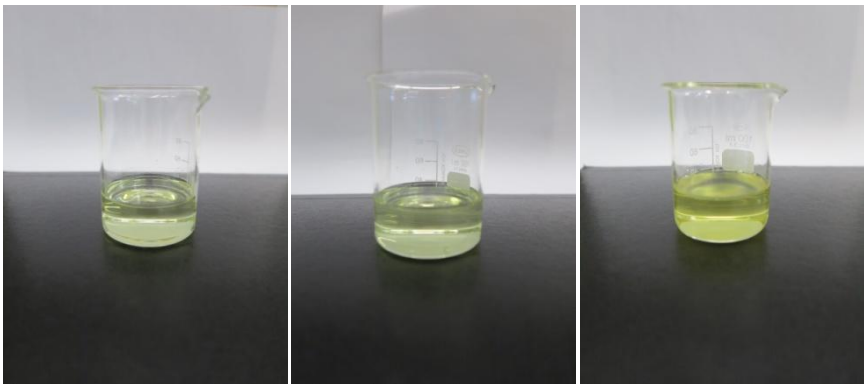

Figure 15. Sunflower oil ethylic biodiesel (left), sunflower oil methylic biodiesel (middle) and waste frying oil ethylic biodiesel (right) after purification.

4.3 Biodiesel characterization and quality analysis

The limit values considered refer to the Portuguese Standard NP EN 14214 (Section 1).

Table 5 shows the results obtained in previous studies for the properties considered of the three types of biodiesel using the studied conditions.

Table 5 - Biodiesel quality parameters obtained for sunflower oil ethylic biodiesel, sunflower oil methylic biodiesel and waste frying oil ethylic biodiesel using the optimized studied conditions. Source: Dias et al. 2008, 2014.

Quality parameter	SEB	WFEB	SMB
EC (wt. %)	98.7 ± 0.9	98.1 ± 1.1	97.6
IV (cg I_2 g^{-1})	124	126	127
KV (mm^2 s^{-1})	4.94 ± 0.01	5.04 ± 0.02	4.57
AV (mg KOH g^{-1})	0.28 ± 0.01	0.94 ± 0.02	0.26
WC (mg kg^{-1})	137 ± 5	160 ± 2	964

The values of the quality parameters for the three types of biodiesel obtained were further compared to the ones found in the mentioned studies.

4.3.1 Water content

Table 6 shows the values of the WC of the three types of biodiesel analyzed.

Table 6 - Water content of sunflower oil ethylic biodiesel, sunflower oil methylic biodiesel and waste frying oil ethylic biodiesel.

Biodiesel	WC (mg kg^{-1})	NP EN 14214
SEB	602 ± 30	< 500 mg kg^{-1}
SMB	454 ± 55	
WFEB	7 786 ± 105	

The results reveal that the purification method was inefficient to lower the WC of both ethylic biodiesel to values in agreement with the standard (< 500 mg kg^{-1}) due to the difficulties during the washing stage mentioned in the previous section. SMB was the only biodiesel produced showing a WC in agreement with the quality standard.

Dias et al. (2014) studied the ethanolysis of SO (WC = 600 mg kg^{-1}) and WFO (WC = 700 mg kg^{-1}) obtaining biodiesel WC values of 160 mg kg^{-1} and 137 mg kg^{-1} for SEB and WFEB, respectively; Felizardo et al. (2006) studied the methanolysis of WFO (WC = 1300 mg kg^{-1}) obtaining a WC of 2100 mg kg^{-1} for the corresponding biodiesel. An optimization study (Antolín et al. 2002) regarding SMB production led to a biodiesel with a WC of 615 mg kg^{-1}.

WC value of both SEB and WFEB were higher than the values of the literature. The high WC value of WFEB might be related to the WFO content in oxidation products that might lead to increased polarity of the matrix molecules which consequently increase the solubility of water in oil and water emulsification

occurrence (Paasimaa 2005). The use of a different purification system could lead to better results since Dias et al. (2014) showed that the ion exchange PD 206 Resin leads to lower WC of SEB.

4.3.2 Composition and content of fatty acid esters

Table 7 shows the percentage distribution of the fatty acid esters present in each biodiesel, and respective purity and IV.

Table 7 - Composition and percentage distribution of the fatty acid esters present in each biodiesel and respective purity and iodine value.

Ester (wt. %)	Biodiesel		
	SEB	SMB	WFEB
C 16: 0	5.56 ± 0.06	5.35 ± 0.02	6.69 ± 0.03
C 18: 0	3.17 ± 0.03	3.21 ± 0.01	3.29 ± 0.03
C 18: 1	47.40 ± 0.03	47.3 ± 0.1	31.02 ± 0.05
C 18: 2	42.7 ± 0.1	42.87 ± 0.07	57.9 ± 0.1
C 20: 0	0.28 ± 0.05	0.254 ± 0.002	-
C 20: 1	0.2 ± 0.1	0.19 ± 0.03	0.246 ± 0.007
C 22: 0	0.7 ± 0.3	0.85 ± 0.02	0.83 ± 0.06
Purity (wt. %)	99.0 ± 0.3	99.9 ± 0.2	93.1 ± 0.7
IV (cg I_2 g^{-1})	114.9 ± 0.2	115.06 ± 0.08	127.2 ± 0.3

The lower reference value for FAE content is 96.5 wt. %, according to the Portuguese Standard NP EN 14214. Only WFEB contains lower purity, and the purity obtained for SEB is slightly lower than that obtained for SMB, but is still higher than the limit value.

According to Dias et al. (2008), the purity was over 95.0 wt. % for SMB and approximately 90.0 wt. % for WFMB. A purity of 98.7 wt. % and 98.1 wt. % for SEB and WFEB, respectively, was also obtained by Dias et al. (2014).

The purity of WFEB was considerably lower than that found by Dias et al. (2014) where 98.1 wt. % of purity was obtained, and also the one found by Felizardo et al. (2006) that obtained a WFMB presenting a purity of 98.0 wt. %, possibly due to the higher production of emulsions during water washing. However, the value was much higher than the biodiesel purity found by Encinar et al. (2007), which was 72.5 wt. %. The values of purity obtained for the SEB and SMB are consistent with literature (Dias et al. 2008, 2014).

The studies analyzed show that the values of purity are consistently higher for sunflower oil biodiesel compared to waste frying oil biodiesel when both are produced through the same route (methylic or ethylic). A relation between the PY of biodiesel and the EC was expected according to Encinar et al. (2007): higher PY values led to higher EC of the biodiesel.

The reason why the IV obtained for SEB and SMB are lower than those found in previous studies (Dias et al. 2008, 2014) relates to the differences between the raw materials composition, even though they are both sunflower virgin oils. The IV`s obtained are similar to those observed in Ramalho et al. study (2011); the IV of the WFEB is consistent with Dias et al. (2014) study. Although the composition of total unsaturated fatty acids of both oils is similar, the WFEB presents a higher IV due to the higher percentage of C18:2. The IV can be reduced by mixing the oil with other types of oil containing less unsaturated fatty acids (e. g. rapeseed oil) (Dias et al. 2008).

Figure 16, 17 and 18 show typical chromatograms of samples corresponding to SMB, SEB and WFEB, respectively.

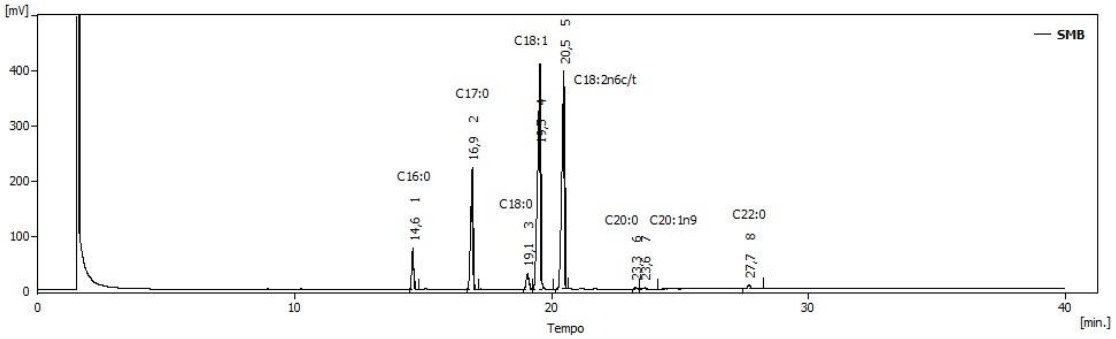

Figure 16. Chromatogram of a sunflower oil methylic biodiesel. C 17:0 is the internal standard.

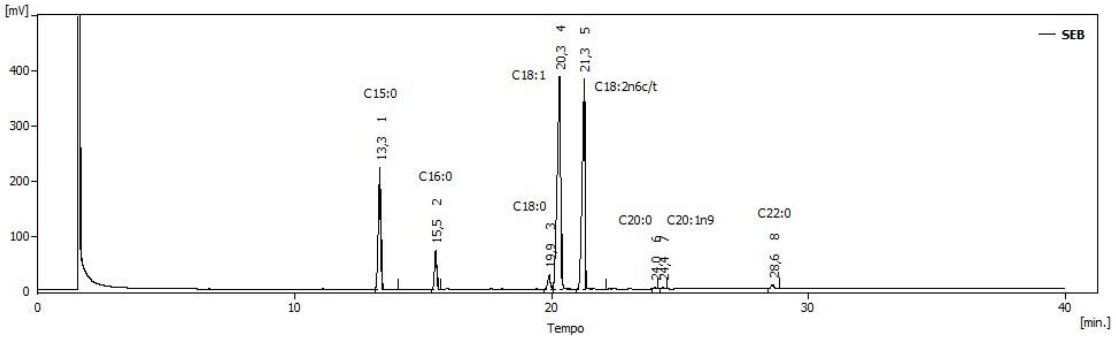

Figure 17. Chromatogram of a sunflower oil ethylic biodiesel. C 15:0 is the internal standard.

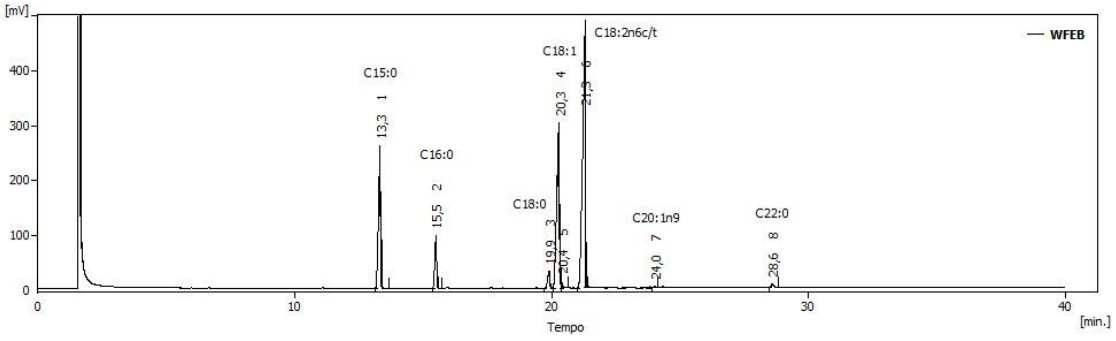

Figure 18. Chromatogram of a waste frying oil ethylic biodiesel. C 15:0 is the internal standard.

4.3.3 Acid value

Table 8 shows the values of the AV parameter of the three types of biodiesel analyzed.

Table 8 - Acid value of sunflower oil ethylic biodiesel, sunflower oil methylic biodiesel and waste frying oil ethylic biodiesel.

Biodiesel	AV (mg KOH g^{-1})	NP EN 14214
SEB	0.55 ± 0.02	< 0.50 mg KOH g^{-1}
SMB	0.16	
WFEB	0.865 ± 0.002	

The combined analysis of Table 3, 6 and 8 shows that both AV and WC of all three types of biodiesel are higher than the AV and WC of the correspondent raw materials.

The maximum limit value for AV of biodiesel is 0.50 mg KOH g^{-1}, according to the Portuguese Standard NP EN 14214. Only the AV of SMB is lower than the limit value. The AV of SEB and WFEB was higher than the one of SMB due to higher impurities (might be FFA), as shown by the lower FAE content (Table 7).

The AV of SEB is higher than the correspondent value referred in Dias et al. (2014) (0.28 mg KOH g^{-1}), the AV of WFEB is close to the value of Dias et al. (2014) study (0.94 mg KOH g^{-1}), and the AV of SMB is lower than the correspondent value of Dias et al. (2008) study (0.26 mg KOH g^{-1}). As in the case of biodiesel produced in the present study, the AV increased in a previous study (Felizardo et al. 2006) that showed that the maximum AV for WFMB produced was 0.47 mg KOH g^{-1} while the samples of treated oils presented an AV of 0.42 mg KOH g^{-1}. Chhetri et al. (2008) showed that the AV of the WFEB was 0.29 mg KOH g^{-1}. Rashid et al. (2009) study resulted in a SMB that, while adopting optimal conditions, showed an AV of around 0.40 KOH g^{-1}. The values of AV obtained for SEB and SMB are similar as expected based on previous studies (Dias et al. 2008, 2014, Ramalho et al. 2011).

4.3.4 Kinematic viscosity at 40 ºC

Table 9 shows the values of the KV of the three types of biodiesel analyzed.

Table 9 - Kinematic viscosity at 40 ºC of sunflower oil ethylic biodiesel, sunflower oil methylic biodiesel and waste frying oil ethylic biodiesel.

Biodiesel	KV ($mm^2 s^{-1}$)	NP EN 14214
SEB	5.08 ± 0.02	3.50-5.00 $mm^2 s^{-1}$
SMB	4.58 ± 0.02	
WFEB	5.28 ± 0.02	

The limit range for the KV is 3.50 to 5.00 $mm^2 s^{-1}$. For this parameter, only SMB viscosity is within the referred limits, while SEB and WFEB viscosities slightly exceed the upper limit.

The KV of SEB and WFEB are slightly higher than the values obtained by Dias et al. (2014) study (4.94 and 5.04 $mm^2 s^{-1}$, respectively), and the KV of SMB is similar to the value obtained by Dias et al. (2008) study (4.57 $mm^2 s^{-1}$). Previous studies about WFEB led to a product with a KV of 4.80 $mm^2 s^{-1}$ (Saifuddin and Chua 2004) and 5.02 $mm^2 s^{-1}$ (Chhetri et al. 2008). Another study (El-Adawy et al. 2013) showed a WFMB containing a kinematic viscosity at 38 ºC of 4.64 $mm^2 s^{-1}$, while the raw material presented a kinematic viscosity at 38 ºC of 12.50 $mm^2 s^{-1}$. The result of the AV obtained during this study regarding WFEB is higher than all the literature referred.

Since viscosity and acid content are related, the higher KV value for SEB might be partially explained by its higher AV when compared to SMB. Higher AV and KV values of WFEB might be due to higher AV of WFO. The KV of both ethylic biodiesel was higher than the KV of the methylic biodiesel, which is consistent with Knothe et al. (2005) study that stated that ethylic biodiesel normally presents higher KV than methylic biodiesel.

All three types of biodiesel showed a similar unsaturation degree, which is not consistent with the results obtained: the two biodiesel with the lowest percentage of unsaturated esters (SEB and SMB) have lower viscosity than WFEB.

The values of KV obtained for SEB and SMB are similar to the ones found in previous studies (Dias et al. 2008, 2014, Ramalho et al. 2011).

An inverse relation between KV and EC was also observed in this study. The same tendency was suggest by previous studies (Filippis et al. 1995, Zhang et al. 1998, Allen et al. 1999, Encinar et al. 2007).

4.3.5 Oxidation stability at 110 ºC

Table 10 shows the values of the OS of the three types of biodiesel analyzed.

Table 10 - Oxidation stability at 110 ºC of sunflower oil ethylic biodiesel, sunflower oil methylic biodiesel and waste frying oil ethylic biodiesel.

Biodiesel	OS (h)	NP EN 14214
SEB	0.31	8 h
SMB	0.50 ± 0.03	
WFEB	1.90 ± 0.02	

The Portuguese Standard NP EN 14214 requires that the OS of biodiesel is higher than 8 h. All biodiesel types produced fall short regarding OS, requiring the addition of antioxidants to be commercialized.

SEB and SMB show lower OS than the SO. Still, WFEB shows higher OS than WFO.

The SMB obtained by Rashid et al. (2009) presented an OS of 2.0 ± 0.1 h. Tavares et al. (2011) obtained a SEB with an OS of 0.75 h. The mentioned

studies led to results indicating a higher OS of the SEB compared to SMB, which relation is verified by the results from Table 7. Rashid et al. (2009) compared the differences between ethylic and methylic biodiesel from poultry fat, and showed that the OS was similar (0.47 h and 0.40 h for ethylic and methylic biodiesel, respectively).

El Sabagh et al. (2011) study obtained WFMB with an OS of 0.40 h; Wang et al. (2010) study obtained a WFMB with an OS of 5.10 h. This shows that higher OS are possible using this raw material, although in the present study the value was lower.

4.4 Biodegradation analysis

4.4.1 Biodiesel alone

Anaerobic batch reactors were setup with SEB, SMB and WFEB in order to determine the methane production from different types of biodiesel made from different sources and alcohols. As described in the previous section, the three types of biodiesel that were produced have different characteristics: both SEB and SMB show high EC, low KV and low WC while WFEB shows a slight lower EC, higher KV and AV, and extremely high WC.

The color of resazurin of the live and autoclaved batch reactors changed through time. All batch reactors were initially pink in color, which indicated the existence of non-anaerobic conditions. After several days, the batch reactors turned clear, which means that anaerobic conditions were present.

Figure 19 shows the production of methane for the three different types of biodiesel, a control (without biodiesel), and an autoclave control. Each data point corresponds to the time when the headspace samples from live bottles were removed and analyzed (days 21, 33, 47 and 61 after setup). The autoclaved controls were analyzed at days 4, 20, 33, 49 and 60.

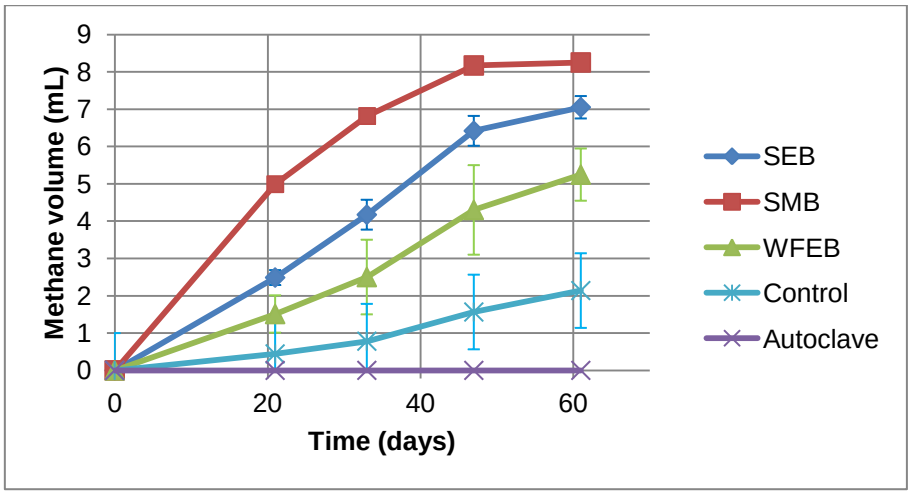

Figure 19. Variation of methane produced (mL) with time (days), since day 0, in the live batch reactors containing the three types of biodiesel.

As can be seen from the graph, methane production was observed after 21 days for all types of biodiesel. Similar behavior was observed in previous studies. Methanogenic conditions (complete consumption of nitrate and sulfate) were reached after 35 days with soybean biodiesel in the study by Corseuil et al. (2011). Borges et al. (2014) observed methane production in methylic biodiesel from soybean oil, castor oil and pork lard oil between 18 and 30 days from the start of the experiment. In addition, the rate of methane production was higher for the SMB, followed by SEB and WFEB. This may be due to the lower KV value of SMB compared to SEB. These results are similar to the study by Corseuil et al. (2011) as approximately 85 % of Soybean Methylic Biodiesel (SbMB) was removed after 45 days, while only 40 % of Castor Methylic Biodiesel (CMB) was removed after 90 days. The authors attributed it to differences in the viscosity between SbMB and CMB, since CMB has higher viscosity and therefore less bioavailability and lower biodegradation.

Another study showed that methane production was similar between SbMB, CMB and Pork Lard Methylic Biodiesel (PLMB) (Borges et al. 2014). However, less than 2 % of the theoretical amount of methane was produced in this study. The authors attributed this result to micro and/or macro-nutrient limitations.

Significant methane production was also observed in the present study in the controls without biodiesel. This is likely due to the large amount of TOC (4.3 %) present in the sediment. Negligible methane production was observed in the autoclaved controls.

The rate of methane production in SMB decreased at day 47 and, by day 61, there was very little methane being produced. In addition, the maximum theoretical amount of methane is 6.1 mL, based on the amount of COD that was added. Taking into account the methane produced from the corresponding control reactor at day 61, the amount of methane produced in SMB approximately equal to the theoretical amount of methane production (Table 11). These results are similar to the ones by Corseuil et al. (2011) study, since 85 % of SMB was removed.

Table 11 - Methane production in the batch reactors (%) compared to the theoretical amount after 61 days of incubation, discounting the methane production from the corresponding control reactor.

Batch	SEB	SMB	WFEB
Biod. + Benz.	59	70	53
Biodiesel	81	100	51

Concerning the other batch reactors, SEB, WFEB and the control bottles are continuously producing methane. At day 61, methane production amounts to 81 % and 51 % of the theoretical maximum (6.1 mL) for SEB and WFEB, respectively (Table 11). Therefore, monitoring will continue to see whether both of these types of biodiesel will reach this value. Only 40 % of CMB was removed after 90 days of monitoring in the study from Corseuil et al. (2011). However, since CMB removal was not decreasing, it is not known whether complete removal would have occurred if monitoring had continued. Estimated COD recoveries for CMB, SMB and PLMB were 40.3 %, 28.9 % and 29.4 %, respectively (Borges et al. 2014).

As previously stated, biodiesel with high viscosity interferes with its biodegradation due to decreasing bioavailability. In addition, the slower biodegradation of WFEB may be related to VFA and LCFA accumulation, since they can significantly reduce methane production (Angelidaki and Ahring 1992, Rinzema et al. 1994, Alves et al. 2009, Eiroa et al. 2012). Future analyses of biodiesel intermediates may provide further insights into the biodegradation behaviors of SMB, SEB and WFEB.

4.4.2 Benzene blended with biodiesel

Experiments were also setup with benzene only and blended with each of the three types of biodiesel in order to examine the impact of biodiesel on benzene. Similar behaviors were observed with respect to methane production and the three types of biodiesel (Figure 20). After 61 days, SMB produced the most, followed by SEB and then WFEB. However, in this case, all three types of biodiesel are continuously producing methane while experiments regarding biodiesel alone led to an appearing depletion of SMB.

Calculations were also done to compare the amount of methane produced to the theoretical maximum and the results can be found in Table 11. In this case though, the methane production from benzene was also excluded (approximately a net of 1 mL after 61 days taking into account a native methane production from the sediment of 2 mL).

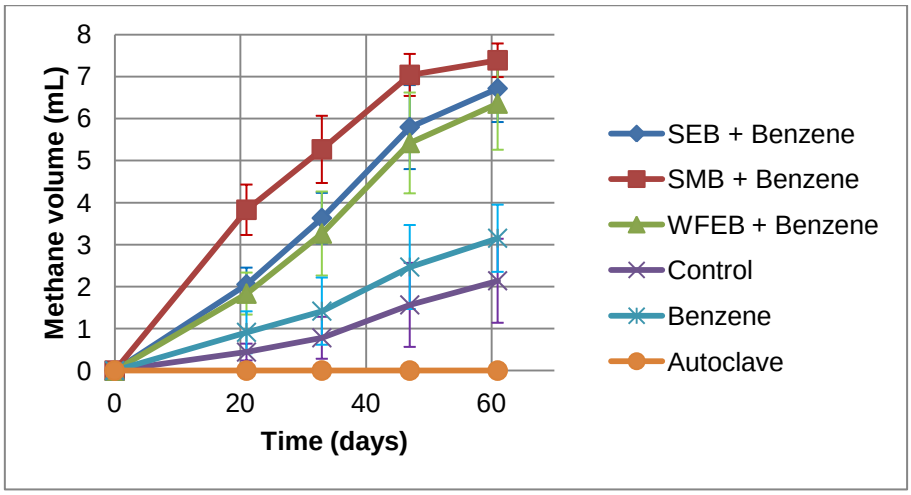

Figure 20. Variation of methane produced (mL) with time (days), since day 0, in the live batch reactors containing the three types of biodiesel blended with benzene.

Comparing the percentage of methane produced to the theoretical amount in both cases (biodiesel alone versus biodiesel + benzene), the amount of methane produced due to biodiesel biodegradation is lower when biodiesel is blended with benzene for SEB and SMB, possibly due to inhibitory effect of benzene and benzene intermediates on biodiesel biodegradation, and to phenomena related to substrate competition. Regarding WFEB, the methane production is approximately the same, which suggests that the presence of benzene does not affect its biodegradation. However, the values in Table 11 could change since methane production for all types of biodiesel are continuing to increase.

Experiments were also monitored for benzene biodegradation (Appendices B6 and B7) and the results for benzene removal in the live reactors, taking into account the benzene removal in the AC, are shown in Table 12. Only one data point between the beginning (day 0) and the end of the experiment (day 61) was included (day 33) since it is approximately at the midpoint of the experiment. This was done due to inconsistencies regarding the other values obtained during monitoring.

Table 12 – Benzene removal (%) in the live batch reactors, at days 33 and 61.

Type of sample	Day 33	Day 61
Benzene	6 ± 6	15 ± 5
SEB + Benzene	4 ± 3	12 ± 5
SMB + Benzene	5 ± 3	9 ± 4
WFEB + Benzene	-4 ± 6	-1 ± 10

For the first 33 days, based on the averages and standard deviations of the different batch reactors, benzene removal in all of the treatments was low and similar to each other. However, the results of day 61 show that, while the average removal increased for all treatments, the large standard deviations of the different treatments indicates the only statistical difference observed was between benzene only and WFEB + benzene.

The negative values of benzene removal obtained for WFEB + benzene batch reactors have no meaning since, in the limit, the removal percentage should be 0 %, which corresponds to a total inhibition of WFEB on benzene biodegradation. This may be explained by sediment heterogeneity. In addition, unexpected carryover between analyses may have also impacted the results for not only WFEB but for all treatments.

Benzene persistence in methanogenic conditions may be due to its low bioavailability and the restricted range of microorganisms capable of its degradation (Carsten et al. 2011). The simultaneous analysis of Tables 11 and 12 confirm that biodiesel is more readily biodegraded under anaerobic conditions than benzene.

The results presented in this work regarding the influence of biodiesel on benzene biodegradation are different than those in previous studies by Corseuil et al. (2011) and Borges et al. (2014). Those studies showed that biodiesel negatively impacted monoaromatic hydrocarbon removal. Complete toluene removal increased from 25 days to 34 days in the presence of soybean biodiesel (Corseuil et al. 2011). Similarly, 45 % of benzene was removed within 34 days with soybean biodiesel while 90 % was removed in its absence

(Corseuil et al. 2011). Approximately 18 % of benzene was removed after 150 days compared to approximately 15 %, 11 % and 7 % in the presence of SbMB, PLMB and CMB, respectively (Borges et al. 2014).

The reason for this impact of biodiesel on benzene removal may lie in the preferential biodegradation of biodiesel, which may lead to the depletion of needed nutrients that will not be available for benzene biodegradation, and the production of inhibitory compounds (Duetz et al. 1994, Silva and Alvarez 2002, Lovanh et al. 2002, Lovanh and Alvarez 2004, Ma et al. 2013).

It is not yet known why the results for benzene removal are different than in those previous studies. However, it should be noted that the results included in this thesis are part of a continuous process that has not finished since methane production is still increasing, while at the same time benzene concentrations are still decreasing. Therefore, the results presented for benzene until day 61 may not be the latest regarding the negative impact of biodiesel on benzene biodegradation. A determination on this will be made once methane production ceases.

5. Conclusions

The present work included the production of three types of biodiesel: two from commercial sunflower oil through a methanolic and ethanolic route, and one from waste frying oil through ethanolic route. Biodiesel production was followed by its characterization.

The three types of biodiesel that were produced showed different characteristics: both sunflower oil ethylic and methylic biodiesel showed high ester content (99.0 ± 0.3 wt. % and 99.9 ± 0.2 wt. %, respectively), lower kinematic viscosity at 40 ºC (5.08 ± 0.02 mm^2 s^{-1} and 4.58 ± 0.02 mm^2 s^{-1}, respectively) and water content (602 ± 30 mg kg^{-1} and 454 ± 55 mg kg^{-1}, respectively) compared to the waste frying oil ethylic biodiesel that showed a slightly lower ethyl ester content (93.1 ± 0.7 wt. %), higher kinematic viscosity at 40 ºC (5.28 ± 0.02 mm^2 s^{-1}) and acid value (0.865 ± 0.002 mg KOH g^{-1}), and extremely higher water content (7 786 ± 105 mg kg^{-1}).

The comparison between the methane production resulting from the biodegradation of the biodiesel during the 61 days of incubation and the theoretical amount suggests that the degradation of sunflower oil methylic biodiesel is close to being completed and that the degradation is mostly due to biological processes. The amount of methane produced due to biodiesel biodegradation was 100 %, 81 % and 51 % of the theoretical amount for sunflower oil methylic biodiesel, sunflower oil ethylic biodiesel and waste frying oil ethylic biodiesel, respectively.

Methane production in studies where biodiesel was blended with benzene showed that less was produced for sunflower oil methylic biodiesel and sunflower oil ethylic biodiesel when compared to the experiments without benzene.

The results regarding the impact of biodiesel on benzene biodegradation showed that more benzene was removed by day 61 compared to day 33. In addition, only waste frying oil ethylic biodiesel showed less methane production

compared to benzene only. However, more monitoring is required in order have a complete picture of the final results.

6. Future recommendations

The present study shows the complexity of the degradation of biodiesel and its impact on benzene degradation. To obtain more reliable and conclusive results, further studies are required, taking into account other factors that may have influenced the results, for instance, the characteristics of the sediment.

Continued biodegradation analysis (currently in progress) is essential to understand the physicochemical and biological phenomena inherent to the study, namely taking into account methane production and to evaluate if it reaches 100 % of the theoretical value, and also benzene degradation.

Also, the fate of different types of biodiesel and their impact on benzene biodegradation should be examined under different redox conditions, namely nitrate, iron/manganese and sulfate reducing conditions.

The determination of intermediate compounds (Long Chain Fatty Acids (LCFA), acetate) should be done in order to evaluate the extent of biodiesel biodegradation and the fate of biodiesel; the relation of such compounds to its physicochemical properties would be of great importance in future studies. Since LCFA are slowly produced, their analysis throughout the incubation period will allow for further understanding of the processes involved.

Finally, an analysis of the microbial communities, population shifts and the relationship between the different biodiesel types would also be extremely useful and should be considered in future studies.

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Appendix A

A1. Standardization of the KOH solution used for acid value determination

$$C_{KOH} = 0.099\ mol/L$$

A2. Formula and molecular weight of fatty acids.

Ester	Molecular formula	Molecular weight (g/mol)
C16: 0	$C_{16}H_{32}O_2$	256.4
C18: 0	$C_{18}H_{36}O_2$	284.5
C18: 1	$C_{18}H_{34}O_2$	282.5
C18: 2	$C_{18}H_{32}O_2$	280.5
C20: 0	$C_{21}H_{42}O_2$	326.6
C20: 1	$C_{21}H_{40}O_2$	324.5
C22: 0	$C_{23}H_{46}O_2$	354.6

Appendix B

B1. Calculation of the theoretical value of Chemical Oxygen Demand (COD)

The COD theoretical was calculated based on the stoichiometry oxidation-reduction reaction of a compound generic represented by $C_xH_yO_z$ (Equation B1) and is expressed by mass per unit of $C_xH_yO_z$ (Equation B2) (Haandel and Lubbe 2012).

$$C_xH_yO_z + {}^{1}/_{4}\,(4x + y - 2z).O_2 \rightarrow x.CO_2 + {}^{y}/_{2}.H_2O \quad \text{(B1)}$$

$$COD_t = 8.\frac{4x+y-2z}{(12x+y+16z)}\ g\ COD.g^{-1}C_xH_yO_z \quad \text{(B2)}$$

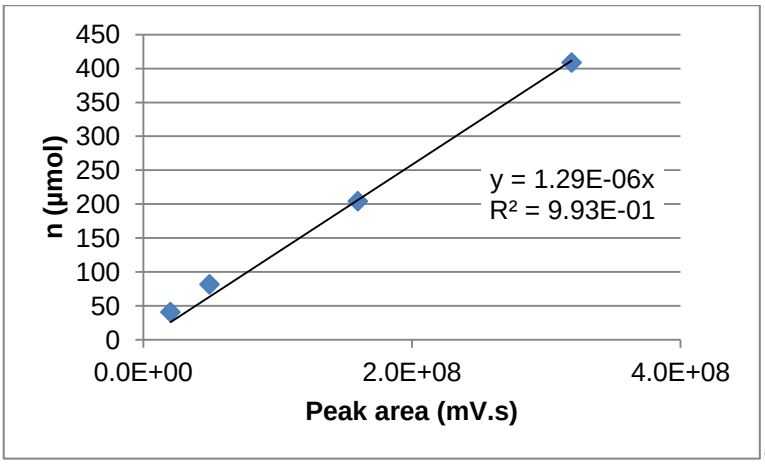
B2. Calculation of the theoretical maximum volume of methane

After calculating the theoretical value of COD following the steps mentioned in Appendix B1, the maximum theoretical value of methane production calculation is based in the assumption that 1 kg of COD produces 0.35 m^3 of methane (Haandel and Lubbe 2012).

B3. Methane calibration curve determination

1. Wash four serum bottles with soap and hot water, and rinse with distilled water. Leave the bottles to air dry or use an oven to promote a complete drying.
2. Add 55 mL of distilled water to each 72 mL bottle, and seal them using a Teflon©-lined septa and an aluminum crimp cap.
3. Add 1, 2, 5 and 10 ml of methane to each of the bottles as follows: Turn on the methane gas cylinder (in F202). Allow the gas to flow through the syringe. Pull in the desired volume of gas and pause a few seconds. Inject the methane into the batch reactor. Immediately invert the bottle.
4. Incubate the bottles in an automatic shaker for 1 to 4 hours.
5. Remove 0.1 mL of headspace gas and inject it onto the GC.
6. The response factor corresponds to the linear regression of the slope. The linear regression (obliged to pass through the origin) relates the number of moles (µmol) of methane with the corresponding peak area (mV.s) determined on the GC (Figure B1). The response factor was then multiplied by a proportion factor in order to correct the calibration curve for the volume of the batch reactors used (72 mL versus 160 mL). This can be done since the same liquid to headspace ratio used in the 72 mL bottles was used in the 160 mL bottles. The table below shows the number of moles added and the corresponding peak area for each 72 mL bottle.

Number of moles (µmol)	Peak area (mV.s)
41	20260360
82	49391127
204	159796779
409	318968358



a

Figure 21. Methane calibration curve using 72 mL batch reactors.

The volume of methane is then determined by the ideal gas equation and is described below (Equation B3).

$$V = \frac{nP}{RT} \text{ (B3)}$$

where n is the number of moles (µmol) of methane per volume unit (L), P is the atmospheric pressure (760 mm Hg), R is the gas constant (63.4 mm Hg mol^{-1} K^{-1}) and T is the room temperature (298 K). For instance, based on the *P*, *R* and *T* listed in the previous sentence, 41 µmol corresponds to a volume of 4 mL.

B4. Benzene calibration curve determination

1. Preparation of the stock solution:
   a. Weigh a 22 mL glass vial with a Teflon©-lined septa and an aluminum crimp cap on a 4-digit balance.
   b. Add between 10 and 11 mL of methanol to the vial, seal and reweigh.
   c. Wash a 100 µL syringe at least ten times with methanol and repeat the procedure this time with distilled water.
   d. Place 15 ml of benzene in a flask and wash the syringe at least 5 times with benzene. Add 100 µL of this compound to the vial and reweigh.
   e. Calculate the mass (g) of benzene present in the standard solution.
2. Wash four serum bottles (72 mL) with soap and hot water, and rinse with distilled water. Leave the bottles to air dry or use an oven to promote a complete drying.
3. Weigh the four bottles in a 4-digit balance. Weigh the same bottles with 55 mL of distilled water. Reweigh the bottles after sealing them with an aluminum crimp cap and a Teflon©-lined septa.
4. Add 25, 50, 75 and 100 µL of stock solution to each bottle and reweigh. Calculate the mass (g) of stock solution added. Place the bottles in an inverted position and incubate them in an automatic shaker for 1 to 4 hours.
5. Remove 0.1 mL of headspace gas and inject it into the gas chromatograph.
6. Calculate the amount of benzene present in batch reactors multiplying the mass (g) of stock solution added times benzene mass (g) / stock solution mass (g).
7. The response factor corresponds to the linear regression slop. The linear regression (obliged to pass through the origin of the reference) relates the number of moles (µmol) of benzene with the corresponding peak area (mV.s), determined on the GC. The response factor was then multiplied by a proportion factor in order to correct the calibration curve for the volume of the batch reactors used (72 mL versus 160 mL).

8. Conversion of number of moles (µmol) in benzene concentration (mg/L) is performed according to Equation B4:

$$C_L = \frac{n}{V_L + H_c.V_G} \quad \text{(B4)}$$

where C_L is the concentration of benzene in the liquid phase, n is the number of moles (mol) of benzene in the batch reactor, which is then converted to mass (g) of benzene using molar mass of benzene (78.1 g/mol), V_L is the volume of liquid (L), H_c is the Henry`s dimensionless constant value (0.22 at 298 K) (EPA 1996) and V_G is the volume of gas (L) (Figure B2). The table below shows the benzene concentration and the corresponding peak area for each 72 mL bottle.

Benzene concentration (mg/L)	Peak area (mV.s)
0.61	112260
1.12	231753
1.63	345509
2.21	467001

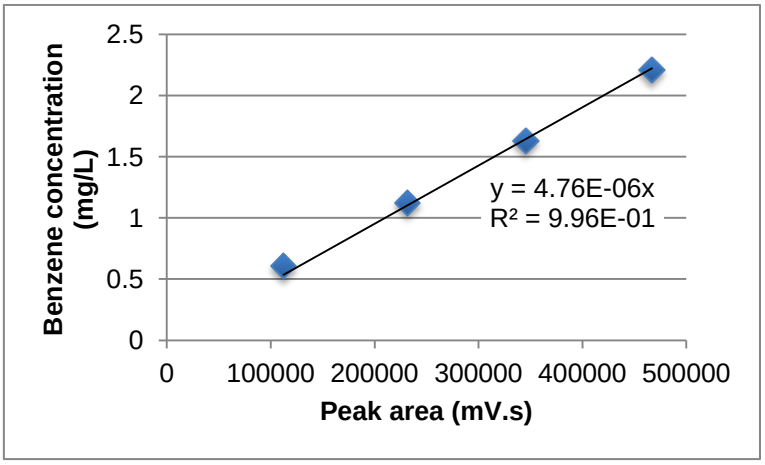


Figure 22. Benzene calibration curve using 72 mL batch reactors.

B5. Basal medium chemical composition

The basal medium used in all experiments was as previously described by Stams et al. 1993, and contained the following (g/L): KH_2PO_4, 0.41; $Na_2HPO_4.2H_20$, 0.53; NH_4Cl, 0.3; $MgCl_2$ $6H_20$, 0.10; $CaCl_2.2H_20$, 0.11; NaCl, 0.3; $NaHCO_3$, 4.0; and $Na_2S.9H_20$, 0.48 as well as acid and alkaline trace elements (each, 1 mL/L) and yeast extract (0.1 mg/L). The acid solution contained (in mM) $FeCl_2$, 7.5; H_3B0_4, 1; $ZnCl_2$, 0.5; $CuCl_2$, 0.1; $MnCl_2$, 0.5; $CoCl_2$, 0.5; $NiCl_2$ 0.1; and HCl, 50. The base solution contained (in mM) Na_2SeO_3, 0.1; Na_2WO_4, 0.1; Na_2MoO_4, 0.1; and NaOH, 10.

B6. Benzene concentration curves for the live batch reactors

Figure B3 shows the behaviour of the benzene concentration when blended with biodiesel. The considered analysis were at days 6, 21, 33, 47 and 61.

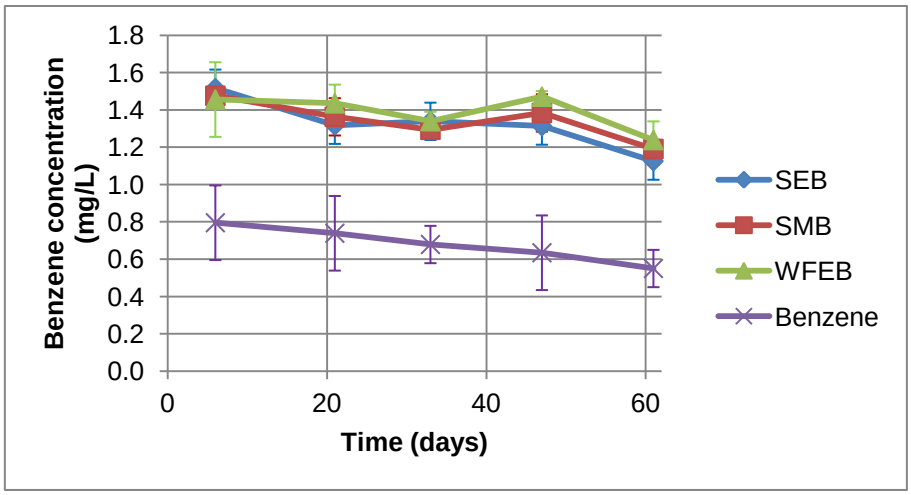


Figure 23. Variation of benzene concentration (mg/L) with time (days), since day 6, in the live batch reactors containing the three types of biodiesel blended with benzene.

B7. Benzene concentration curves for the autoclaved batch reactors

Figure B4 shows the depletion of benzene blended with biodiesel for the autoclave batch reactors controls. The considered analysis were at days 4, 20, 33, 49 and 60.

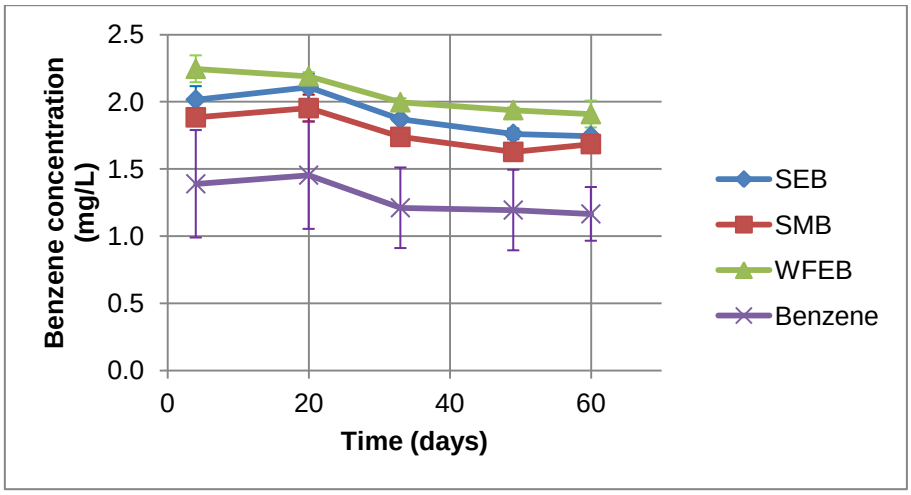


Figure 24. Variation of benzene concentration (mg/L) with time (days), since day 4, in the autoclave batch reactors containing the three types of biodiesel blended with benzene.